MULTIPLE LARGE DNA MOLECULES OF *Azospirillum*

BY

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL
OF THE UNIVERSITY OF FLORIDA IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1982

ACKNOWLEDGEMENTS

The author would like to thank his committee members, Drs. Dennis E. Duggan, Francis C. Davis, Jr., Philip J. Laipis, William W. Hauswirth and L.O. Ingram for their valuable suggestions and criticisms.

He would also like to extend special thanks to Dr. William B. Gurley for help with the hybridization experiments and for the use of his laboratory equipment and supplies.

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May 1982

Chairman: Dennis E. Duggan
Major Department: Microbiology and Cell Science

Six strains of Azospirillum brasilense and two of A. lipoferum were found to harbor as many as eight different-sized circular DNA molecules ranging from 45 to 1500 megadaltons. Identification and separation of these very large molecules were achieved by gently lysing bacteria in the wells of vertical agarose gels, subjecting the lysate to electrophoresis at 2 mA for 6 h, and then continuing electrophoresis at 15-30 mA for an additional 12-48 h. Optimal recovery required lysis at 4°C in the presence of ribonuclease. The technique has been used to isolate large DNAs from other bacteria, including the chromosomes of Escherichia coli and Agrobacterium tumefaciens.

Several types of mutants were isolated from acridine orange-treated cultures of A. lipoferum and A. brasilense. Mutants displaying increased sensitivity to cadmium and

unable to grow on carbon-free media or on ethanol were all found to have lost a specific plasmid. One of these strains was shown to have suffered deletions in most of its remaining DNA molecules. A mutant unable to grow on N_2 or reduce acetylene was isolated from the multiply-deleted strain, but its DNA molecules showed the same electrophoretic mobilities as those of its parent strain. Methionine-requiring auxotrophs, isolated at a high frequency from A. lipoferum cultures, also possessed DNA molecules with unaltered mobilities.

Attempts were made to determine which Azospirillum DNA molecule includes the genes controlling nitrogen fixation by hybridizing a labeled recombinant probe to Southern blots of wild type and mutant DNA molecules. The limited success acheived with this technique indicates that the structural genes for nitrogenase are carried on the largest Azospirillum DNA molecule.

CHAPTER I
OCCURRENCE OF MULTIPLE LARGE DNA MOLECULES IN *Azospirillum* AND A METHOD FOR ISOLATING THEM ON AGAROSE GELS

Introduction

The genus *Azospirillum* comprises Gram-negative, free-living, nitrogen-fixing bacteria found in association with roots of cereal crops and tropical forage grasses (19). Field experiments conducted at the University of Florida showed higher yields of dry matter in *Azospirillum*-inoculated pearl millet and guinea grass than in uninoculated controls (64). More recently, *Azospirillum* inoculation has been reported to enhance corn yields in Israel (46).

The potential agronomic value of this association has prompted studies of carbon and nitrogen metabolism in *Azospirillum* (2, 21, 27, 28, 38, 43, 44, 45, 48, 49, 51), and the scores of strains isolated from various parts of the world have been grouped, on the basis of DNA homology and biochemical characteristics, into two species, *A. lipoferum* and *A. brasilense* (67). Very little is known, however, concerning the genetics of *Azospirillum* (26, 41, 55). In particular, no system of genetic transfer exists which would permit location of the genes controlling nitrogen fixation and facilitate studies of their expression.

Our interest in developing a system of genetic transfer for Azospirillum led us to examine several wild type strains for the presence of plasmids. Our initial attempt to identify plasmid DNA in Azospirillum (9) involved dye bouyant density ultracentrifugation of alkaline denatured lysates (61) and direct visualization of plasmids in the satellite bands by electron microscopy (18). Open circular (OC) DNAs of various contour lengths were seen, but the apparent multiplicity of molecules in each strain and their large sizes relative to the plasmid chosen as a size standard ($Col E_1$) made it difficult to accurately assess the numbers and sizes of plasmids in any given strain. However, plasmids with molecular weights in excess of 300 megadaltons (Mdal) did appear to be present in several Azospirillum strains.

This observation prompted us to try two electrophoretic techniques specifically designed for the isolation of large plasmids (10, 29), but neither of these permitted the isolation of more than three plasmids from any Azospirillum strain. We felt there was a strong possibility that very large plasmids were present in these strains but that they were being sheared during the mechanical manipulations, however gentle, inherent in these procedures.

Therefore we adopted a method which is theoretically the most gentle of all, that described by Eckhardt (22). This technique differs fundamentally from other electrophoretic techniques in that plasmid DNA is not extracted from cells

prior to electrophoresis. Rather, the bacteria are lysed directly in the wells of the gel apparatus, resulting in minimal nicking or breakage of covalently closed circular (CCC) DNA. Using a modified version of this technique, we have discovered the widespread occurrence of a multiplicity of very large DNA molecules in strains of Azospirillum.

The present communication describes the electrophoretic conditions necessary for the successful isolation of these Azospirillum DNA molecules. Estimates of the sizes of the molecules harbored by one Azospirillum strain are provided, based on a comparison of their mobilities with those of plasmids of known molecular weight. The very low mobilities of some of the Azospirillum molecules suggest that they represent small chromosomes rather than large plasmids. Indeed, we have been able to isolate slowly-migrating DNA bands from strains of Escherichia coli and Agrobacterium tumefaciens, including two strains which do not harbor plasmids. Evidence is provided indicating that the slowly-migrating DNA bands isolated from Azospirillum represent CCC DNA uncomplexed with protein.

Materials and Methods

Bacterial strains. Table 1-1 lists the Azospirillum strains examined for plasmid content. Table 1-2 lists other bacterial strains harboring plasmids of known molecular weights used for construction of the standard size curve.

Growth conditions. All bacteria were grown to early stationary phase prior to harvesting for electrophoresis. Azospirillum and A. tumefaciens strains were grown in a succinate/mineral salts medium (50) supplemented with 0.01% yeast extract. Pseudomonas putida and E. coli strains were grown in nutrient broth (Difco) supplemented with 0.01% yeast extract. Growth temperatures were 35°C for Azospirillum and E. coli and 28°C for A. tumefaciens and P. putida.

Plasmid isolation and agarose gel electrophoresis. We used an electrophoretic method based on that described by Eckhardt (22). The protocol outlined here includes modifications found to be necessary for optimal, reproducible visualization of the electrophoretic bands representing the largest DNA molecules.

Vertical gels were cast with 0.6% or 0.7% molten agarose (BioRad Standard Low -m_r) in Tris/borate/EDTA electrophoresis (E) buffer (40). The agarose was tempered at 42°C for 20 min prior to casting the gel in order to minimize contracture during solidification. The plastic comb used to form wells had 16 teeth (13x9x1.5 mm). The gel was submerged in E buffer and allowed to age at least 4 h at 4°C prior to removal of the comb. Spraying the comb lightly with PAM (Boyle-Midway) prevented agarose from sticking to it during removal.

Except where otherwise noted, wells were loaded in the following manner. One milliliter of cell culture was centrifuged for one minute in a microcentrifuge (Fisher Model 235). The

supernatant solution was pipetted off using a vacuum aspirator and the pellet was resuspended in 20-100 µl of 20% ficoll in E buffer. Ten microliter aliquots of the cell suspensions were added to wells preloaded with 15 µl of a solution containing 20% ficoll, 10 µg/ml lysozyme (Sigma), 100 µg/ml ribonuclease A (Sigma), and 0.05% xylene cyanol FF (Kodak) in E buffer. No attempt was made to mix the cell suspension with the lysozyme solution inside the well. The cells were allowed to interact with the lysozyme mixture at 4°C for a minimum of 30 min, and then 30 µl of 10% ficoll, 1% SDS in E buffer was added, followed by 50 µl of 5% ficoll, 1% SDS in E buffer.

A current of 2 mA was applied for a minimum of 6 h, and then the current was raised to 15-30 mA (50-100 V at 4°C). Electrophoresis was continued for 12-48 h depending on the voltage used and the degree of molecular separation required. The large capacity (2.5 l) of the buffer reservoirs of our electrophoresis apparatus made recirculation of buffer unnecessary.

Photography. Gels were stained for a minimum of 30 min in 0.5 µg/ml ethidium bromide and visualized with either a 254 nm hand-held UV light (UV Products) or a 300 nm transilluminator (Fotodyne). Photographs were taken through #4 and #29 Wratten filters, using Type 57 film (Polaroid).

Standard curve construction. Mobility data from 19 gels were normalized to the mobility data of the gel illustrated in Fig. 1-6 according to the method described by Hansen and Olsen (29, Appendix A) except that absolute mobilities

(distances of plasmid migration from origin) rather than relative mobilities (absolute mobilities divided by gel lengths) were used in the calculations (see Appendix A). The number of DNA bands in common between the normalized and standard gels ranged from 7 to 15 with an average of 12. The logarithm of the average normalized mobility of a given standard plasmid was plotted against the logarithm of the molecular weight of that plasmid, and a least squares regression line was calculated (see Appendix B). This regression line was then used to estimate the molecular weights of the Azospirillum molecules as well as the presumed chromosomes of E. coli and A. tumefaciens.

Plasmid nomenclature. The molecule having the lowest mobility in each Azospirillum strain has been designated pAZ1; molecules are then numbered in order of increasing mobility. All the molecules of a given strain are suffixed with that strain designation. For example, the smallest plasmid of Sp13t is pAZ6-Sp13t.

Results

Multiplicity of DNA molecules found in Azospirillum strains. Figures 1-1 and 1-2 illustrate the electrophoretic banding patterns obtained from eight geographically diverse isolates of A. brasilense and A. lipoferum lysed in the wells of vertical agarose gels. Each strain has a characteristic array of DNA molecules of various mobilities, an observation which can be used for purposes of identification. Every strain harbors two molecules whose extremely low

mobilities suggest that they represent small chromosomes rather than plasmids. Under optimal conditions, the recovery of these DNAs is highly reproducible except for the small plasmid which bands in the region of linear DNA in some gels (Fig. 1-1, lane D; Fig. 1-2, lanes C and D).

A comparison of Figs. 1-1 and 1-2 indicates that very long periods of electrophoresis are necessary in order to achieve separation of all the DNA bands, in accordance with what would be expected for very large DNAs. The increase in resolution achieved by increasing electrophoresis time is, unfortunately, accompanied by a tendency for the most slowly-migrating bands to become faint or disappear altogether (data not shown). This suggests, however, that the material is those bands is fragile, presumably because of its high molecular weight.

Effect of ultraviolet light on mobilities of $JM125A_2$ molecules. Figure 1-3 compares the mobilities of UV-irradiated and unirradiated molecules of $JM125A_2$. The $JM125A_2$ molecules were isolated in the usual manner except that electrophoresis was terminated after 4 h at 80 V. Blocks of agar were cut from lanes of the gel, extending from the well to the position of the tracking dye. One of the unstained agar blocks (lane B) was subjected to a dose of approximately 3000 J/m^2 of 254 nm UV, while the other (lane A) was untreated. This dose should have been sufficient to introduce at least one chain break into every CCC DNA molecule in the irradiated gel (6, 7, 33, 56). Both treated and untreated agarose

blocks were then imbedded in a horizontal agarose gel and subjected to electrophoresis for 8 h at 50 V. As indicated in Fig. 1-3, UV irradiation converted the DNA molecules from forms capable of movement through an agarose gel into forms incapable of such movement. Presumably, this represents the conversion of CCC DNA into OC DNA.

Effects of enzymatic treatments on DNA recovery. In order to acquire information concerning the physical relationships of the DNA molecules to other cellular components, the roles of lysozyme, RNase, and protease (Sigma Type VI) in optimal DNA recovery were assessed. Figure 1-4 shows that neither the addition of protease to the standard cell mixture nor the elimination of lysozyme had an appreciable effect on DNA recovery or mobility. The elimination of RNase, however, resulted in failure to recover pAZ1 and pAZ2 as well as poor recovery of pAZ3.

Effect of cell mass on DNA isolation. Figure 1-5 illustrates the result of an experiment in which cell suspensions of JM125A$_2$ and AT181 were serially diluted prior to loading the wells. The smearing of the bands in lanes A and E appears to be due to overloading. The use of only 10^7 cells (lanes D and H) allowed visualization of all the DNA bands in this experiment. However, in other experiments (data not shown) using 10^7 cells resulted in very faint bands, particularly for the smaller molecules. Optimal recovery was usually achieved with 10^8 cells.

Size estimates of DNA molecules. The mobilities of the DNA molecules of JM125A$_2$ in relation to plasmids of known molecular weight and slowly-migrating DNAs of other bacteria are illustrated in Figs. 1-6, 1-7, and 1-8. Based on Fig. 1-6, JM125A$_2$ appears to harbor five molecules larger than the largest standard molecule (pMG1, 312 Mdal). Recovery of the larger JM125A$_2$ molecules was poor in Figs. 1-6 and 1-7; these are included primarily to show the slowly-migrating DNAs isolated from E. coli (Fig. 1-7, lane F; Fig 1-8, lanes E, G, and H) and A. tumefaciens (Fig. 1-7, lane A). The two slowly-migrating DNAs recovered from A. tumefaciens have been given the designations pXX1-AT181 and pXX2-AT181. The isolation of these slowly-migrating DNAs is not completely reproducible (Fig. 1-6, lanes E and F; Fig 1-7, lane E; Fig. 1-8, lanes A and D). Indeed, the difficulty of isolating the molecules appears, in our experience, to be inversely related to mobility. Thus, our rate of success in isolating slowly-migrating DNAs from Azospirillum is 90% or better, but our success rate with the slowly-migrating E. coli DNA has never exceeded 50%. We have never isolated a slowly-migrating DNA band from either of the two Pseudomonas strains used in the present study. This failure may be related to an observed tendency for these strains to lyse prematurely.

Mobility data from 20 agarose gels were normalized and used to construct a standard curve relating electrophoretic mobility and molecular weight (Fig. 1-9). Since reports in the literature had suggested that CCC DNAs larger than 80

Mdal (72) or 140 Mdal (29) migrate faster than predicted from linear extrapolations of standard curves based on smaller CCC DNAs, we initially calculated a regression line not including pMG1 and pMG5. When this line was used to estimate the sizes of the Azospirillum molecules, however, it seemed impossible that the values obtained could be underestimates. We therefore recalculated regression data with the large Pseudomonas plasmids included, and again with the slowly-migrating band from E. coli included and assigned a molecular weight of 2800 Mdal (8,14,28). The three sets of regression line estimates are summarized in Table 1-3. Figure 1-9 is a graph of regression line B, chosen because it includes only those molecules measured by electron microscopic contour length.

Effect of voltage gradient on regression estimates. If the larger molecules examined in the present study were really migrating faster than predicted, this effect should be more pronounced at higher than at lower voltages (24). We were particularly interested in this possibility since, in our attempts to optimize electrophoresis conditions, we had used voltages ranging from 50 V to 100 V and we wished to include data from as many gels as possible in our regression line calculations. Figure 1-10 compares the mobilities of five standard plasmids run at 50 V for 42 h with mobilities of the same plasmids run at 100 V for 24 h. The two curves are nearly parallel and neither displays a convincing change

of slope above 140 Mdal. Table 1-4 indicates that, for each standard plasmid, the ratio of mobility at 100 V to mobility at 50V is a constant value.

Discussion

Eckhardt first described *in situ* lysis of bacteria in agarose gels as a rapid method for plasmid isolation (22). We have sacrificed the rapidity of the technique but exploited its gentleness in order to isolate very high molecular weight CCC DNAs from *Azospirillum* and other bacteria.

Our initial excitement in isolating slowly-migrating DNAs on agarose gels was tempered with concern that the low mobilities might reflect an open circular nature or some protein interaction rather than large size. The electrophoretic behavior of the JM125A$_2$ molecules subsequent to UV irradiation, however, strongly indicates that they are covalently closed and supercoiled. A DNA/protein interaction, while not strictly ruled out by the failure of protease to alter the electrophoretic mobilities of the molecules, seems unlikely in view of this result. Furthermore, such an association would have to be resistant to dissociation by the SDS which quickly migrates from the upper ficoll layers down through the DNA-containing region of the gel during electrophoresis. Thus, the slowly-migrating bands do not appear to represent relaxation complexes of the type isolated from plasmid-bearing strains of *E. coli*, which dissociate yielding OC DNA when exposed to SDS or protease (13).

A comparison of the DNA molecules isolated from A. brasilense strains Sp7 and Sp13t provides further evidence that the slowly-migrating DNAs are not simply isomeric forms of smaller plasmids. Since these two strains were isolated from the same region of Brazil and display nearly identical electrophoretic banding patters, there is a good possibility that they are isogenic except for the occurrence of pAZ6-Sp7 in one strain. If so, none of the slowly-migrating DNAs of either strain could represent an isomeric form of this relatively small molecule.

The requirement of RNase treatment for isolation of the larger Azospirillum DNAs suggests that, in their native forms, these molecules are attached via RNA to some cellular component in a manner precluding entry into the gel matrix. It is also possible that the larger Azospirillum molecules are attached to one another via RNA. Assuming the likelihood that essential genes are carried on the largest two or three molecules, some mechanism to ensure cosegregation of newly-replicated molecules into daughter cells would appear to be necessary. Molecules as large as pAZ1 and pAZ2 might further be expected to exist inside the cell in condensed, folded states. These considerations lead us to postulate that pAZ1 and pAZ2 (and perhaps pAZ3) are arranged in a chromosomal structure closely resembling that believed to occur in E. coli (54, 73, 74). The only difference between the two "nucleoid" structures would be that in E. coli the RNA-stabilized domains comprise a single, continuous DNA molecule,

whereas in Azospirillum these domains are divided into two or three continuous DNA molecules. The remaining Azospirillum molecules might form nonintegrative associations with the Azospirillum nucleoid analogous to those described between other large, stringently controlled plasmids and their host chromosomes (34, 35).

We have attempted to estimate the sizes of the DNA molecules of one Azospirillum strain ($JM125A_2$) by comparing their mobilities to those of plasmids whose sizes have been calculated from electron microscopic contour length measurements. A problem with this effort arose in that five of the $JM125A_2$ molecules migrated more slowly than pMG1, the largest standard plasmid available. Their sizes, therefore, had to be estimated from a linear extrapolation of our standard curve (Fig. 1-9) and so must be considered only approximate.

Some investigators have cautioned against standard curve extrapolations on the grounds that CCC DNAs larger than 80 Mdal (72) or 140 Mdal (29) migrate faster than predicted. For several reasons, this appears not to be the case under our electrophoresis conditions. First, it is difficult to believe that the calculated values for the larger Azospirillum molecules could be underestimates. Second, these estimates change only modestly when the Pseudomonas plasmids are disallowed or when the chromosome of E. coli is included in the regression line calculation (Table 1-4). Third, the hypothetical nonlinearity of the standard curve should have been greater for a gel run at 100

V than for a gel run at 50V, but Table 1-4 indicates that the relative mobilities of the standard plasmids were nearly identical at the two voltages. Finally, a theoretical justification for nonlinearity of standard curves for CCC DNAs in the high molecular weight range has not been advanced. The explanation offered for the fast mobilities of high molecular weight linear DNAs, i.e., "end-on" migration (1, 25), would seemingly not apply to high molecular weight CCC DNA.

From the estimated sizes of the Azospirillum molecules and assuming one copy of each per cell, the full genetic complement of DNA for these bacteria appears to be approximately 4.3×10^9 daltons, some 50% greater than the corresponding value for E. coli (8, 14, 28). At present, we can only speculate as to the reason for this discrepancy. The large complement of DNA may simply reflect the metabolic diversity of these bacteria; Azospirillum species are capable of carrying out most of the known nitrogen transformations (19, 44, 45), can grow heterotrophically (49) or autotrophically (60), and tolerate the full range of oxygen tensions from fully aerobic (49) to anaerobic with nitrate as terminal electron acceptor (45). Alternatively, some of the Azospirillum DNA may be redundant. This redundancy, if it does occur, could provide a basis for recombination among Azospirillum DNA molecules, underlying a potential mechanism for the evolution of new strains.

The application of the modified Eckhardt technique to bacteria harboring size standard plasmids led to the discovery

that slowly-migrating DNAs could be isolated from species other than Azospirillum. Molecules with apparent molecular weights of 500 and 1800 Mdal were isolated from A. tumefaciens AT181 along with the two previously described plasmids. These four molecules may well represent the full genetic complement of this A. tumefaciens strain since the sum of their estimated sizes is 2.6×10^9 daltons. Hence, pXX1-AT181 may, in fact, represent the Agrobacterium chromosome. The slowly-migrating DNA isolated from E. coli strains appears to represent the E. coli chromosome since it displayed an appropriate mobility and could be recovered from both plasmid-harboring and plasmidless strains. Isolation of intact E. coli chromosomes by ultracentrifugation of gently lysed cells through neutral sucrose gradients has been described by others (66, 73).

In summary, we have demonstrated that strains of Azospirillum harbor unique arrays of large DNA molecules. The probability that these molecules comprise the full genetic complement of their host bacteria suggests that Azospirillum should be considered a multichromosomal prokaryote. Arrangement of genetic material in this fashion contrasts sharply with the situation in E. coli, in which more than 90% of the DNA is carried on a single large molecule. The common assumption that the DNA of most prokaryotes is arranged as it is in E. coli may reflect, to a certain extent, the previous lack of a suitable protocol for isolating CCC DNAs larger than 500 Mdal.

Table 1-1. Geographic origins of *Azospirillum* strains

Species	Strain	Isolated by[a]	Place[a]	Country[a]
A. brasilense	Sp7	J. Dobereiner	Rio de Janeiro	Brazil
"	Sp13t	"	"	"
"	Sp84	"	"	"
"	Cd	D. L. Eskew	Riverside, CA	USA
"	$JM82A_1$	J. R. Milam		Venezuela
"	$JM125A_2$	"	Gainesville, FL	USA
A. lipoferum	SpUSA5b	J. Dobereiner	Pullmann, WA	"
"	SpRG6xx	"	Passo Fundo	Brazil

[a]from Reference 67

Table 1-2. Strains containing plasmids of known molecular weight

Species	Strain	Source	Plasmid	MW[a] (ref.)
Escherichia coli K12	C600	this lab	RP4	36 (40)
''	X 1254	''	F14	202 (47)
''	''	''	F	61 (47)
''	''	''	F14 Δ F	141 (47)
''	JC182	B. Bachmann	none	
Escherichia coli C		W. Klipp	none	
Pseudomonas putida	PpS1239	J. Shapiro	pMG1	312 (29)
''	PpS1240	''	pMG5	280 (29)
Agrobacterium tumefaciens	AT181	W. Gurley	pTi-AT181	114 (16)
''	''	''	pAT-AT181	158 (16)

[a]in megadaltons

Table 1-3. Linear regression estimates of sizes of DNA molecules

DNA molecule	A[a]	B[b]	C[c]
pAZ1-JM125A$_2$	1380±25	1510±290	1330±240
pAZ2-JM125A$_2$	1130±180	1230±190	1090±170
pAZ3-JM125A$_2$	600±56	637±62	587±53
pAZ4-JM125A$_2$	385±30	404±32	379±28
pAZ5-JM125A$_2$	345±26	362±30	342±25
pAZ6-JM125A$_2$	128±6	131±6	130±6
pAZ7-JM125A$_2$	46±6	46±6	47±7
pMG1	289±46	302±49	287±46
pMG5	260±35	270±38	259±35
pTi-AT181	113±17	114±18	114±17
pAT-AT181	154±19	158±19	155±18
pXX1-AT181	1650±160	1810±180	1580±150
pXX2-AT181	473±42	500±45	465±40
RP4	33±6	32±6	34±6
F	64±10	64±10	66±10
F14	205±16	212±17	205±16
F14 ΔF	141±15	144±16	142±15
E. coli chromosome	3150±530	3500±610	2970±490

[a]based on mobilities of standard plasmids smaller than 160 Mdal

[b]based on mobilities of all standard plasmids

[c]based on mobilities of all standard plasmids plus E. coli chromosome

NOTE: Sizes are in megadaltons, ± 1s.d.

Table 1-4. Comparison of mobilities of standard plasmids run at 50 V and 100 V

Plasmid	Gel A[a] Mobility (cm)	Gel B[b]	B/A
pMG1	3.10	3.80	1.23
pMG5	3.20	3.90	1.22
pTi-AT181	3.90	4.75	1.22
pAT-AT181	3.50	4.35	1.24
RP4	5.35	6.80	1.27

[a]50 V for 42 h

[b]100 V for 24 h

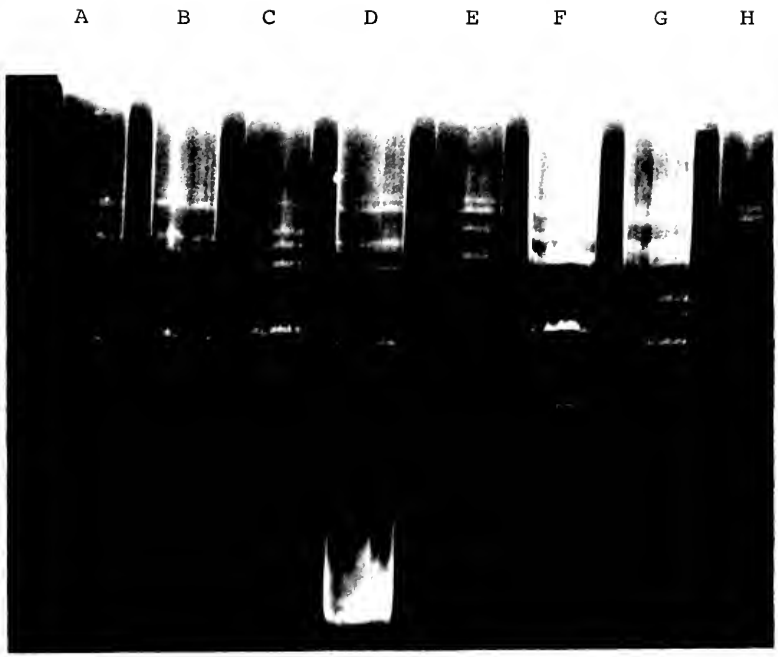


Fig. 1-1. Agarose gel electrophoresis of large DNA molecules from eight Azospirillum strains of diverse geographic origin. Electrophoresis was for 24 h at 80 V. A. brasilense strains: (A) Sp7; (B) Sp13t; (C) Sp84; (D) Cd; (E) JM82A$_1$; (F) JM125A$_2$. A. lipoferum strains: (G) SpUSA5b; (H) SpRG6xx.

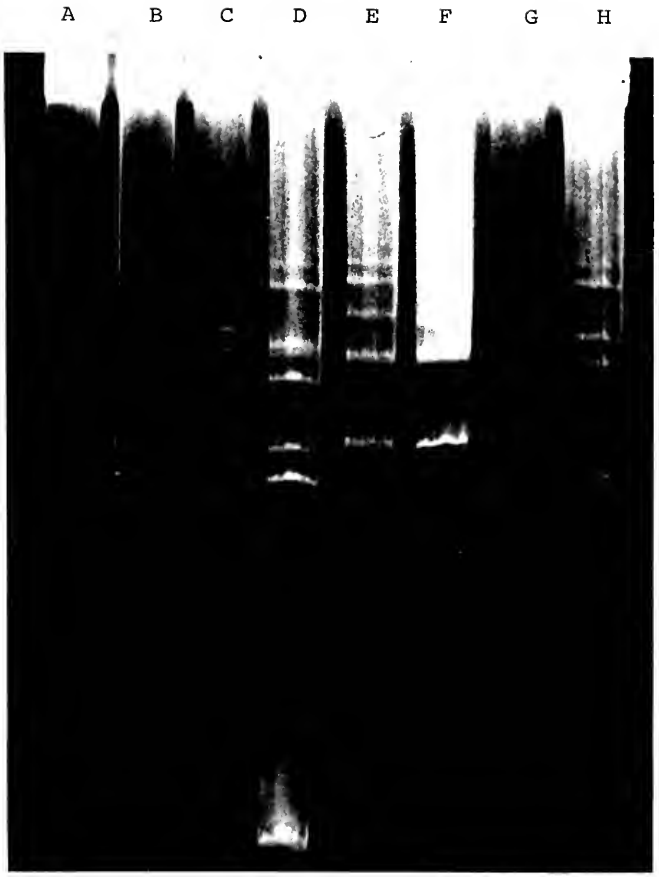


Fig. 1-2. Increased separation of large Azospirillum DNA molecules in an agarose gel subjected to electrophoresis for 36 h at 80 V. A. brasilense strains: (A) Sp7; (B) Sp13t; (C) Sp84; (D) Cd; (E) JM82A$_1$; (F) JM125A$_2$. A. lipoferum strains: (G) SpUSA5b; (H) SpRG6xx.

Fig. 1-3. Agarose gel showing the effect of UV light on mobilities of DNA molecules from JM125A$_2$. Lanes from a preparative vertical gel run at 80 V for 4 h were excised and either (A) not treated or (B) irradiated with 3000 J/m^2 254 nm UV. The agarose blocks were then embedded in a horizontal gel and subjected to electrophoresis at 50 V for an additional 8 h.

Fig. 1-4. Effects of various enzymatic treatments on recovery of DNA molecules from JM125A$_2$. A cell suspension was prepared as usual and 10 ul aliquots were added to wells preloaded with (A) and (B) standard reaction mixture; (C) standard reaction mixture lacking RNase; (D) standard reaction mixture lacking lysozyme. After 1 h, 30 ul 20 mg/ml protease, 20 °/₀ ficoll in E buffer was added to well (B). The reaction mixtures were then allowed to interact an additional 1 h at 4°C. Electrophoresis was for 42 h at 80 V.

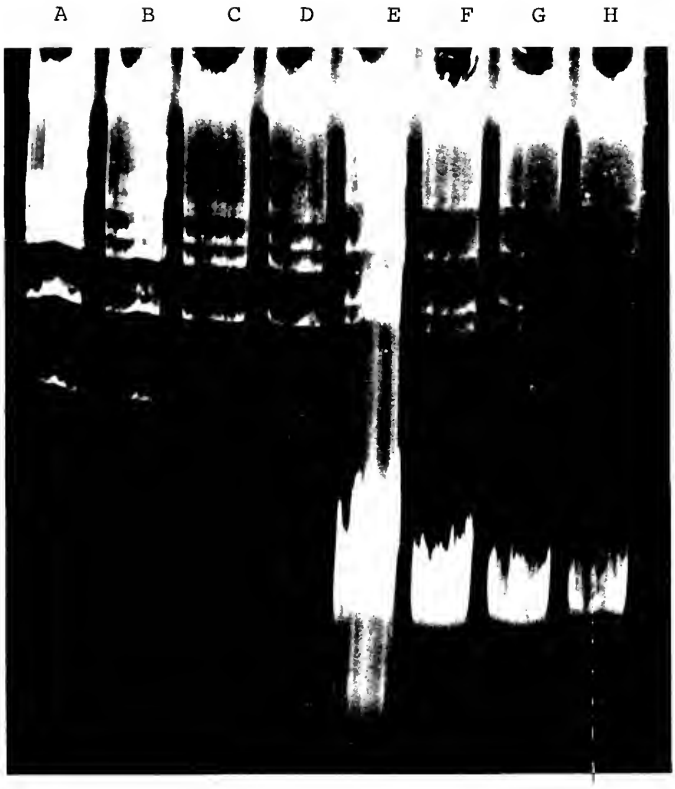


Fig. 1-5. Effect of cell number on isolation of DNA molecules from (A-D) JM125A$_2$ and (E-H) AT181. Electrophoresis was at 80 V for 24 h. (A) and (E) $3x10^8$ cells; (B) and (F) 10^8 cells; (C) and (G) $3x10^7$ cells; (D) and (H) 10^7 cells.

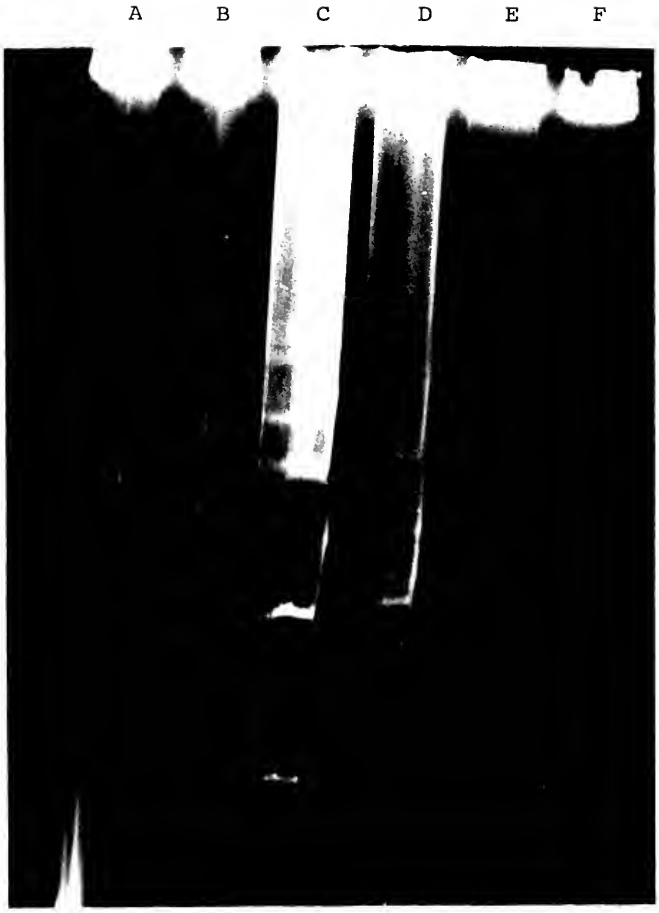
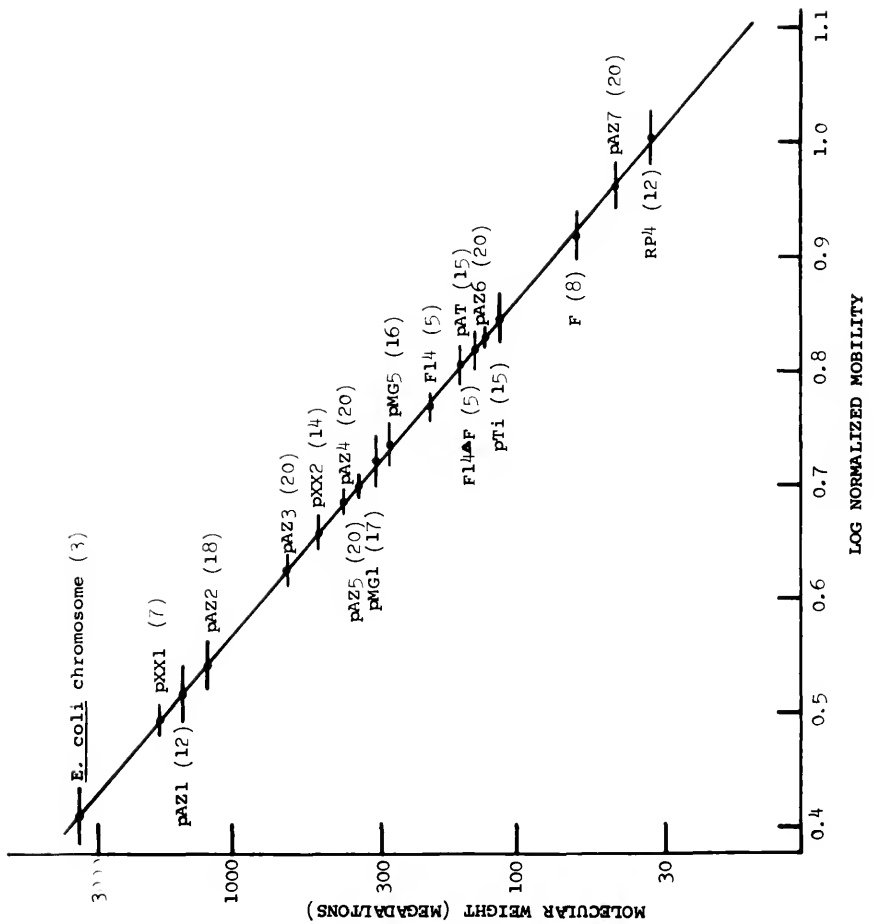


Fig. 1-6. Agarose gel electrophoresis showing mobilities of JM125A$_2$ DNA molecules in relation to mobilities of size standard plasmids. Electrophoresis was at 90 V for 28 h. (A) PpS1239 (pMG1); (B) PpS1240 (pMG5); (C) JM125A$_2$; (D) AT181 (pTi,pAT); (E) C600 (RP4) (F) X1254 (F).

Fig. 1-7. Agarose gel electrophoresis showing slowly-migrating DNAs isolated from A. tumefaciens and E. coli. Electrophoresis was for 24 h at 75 V. (A) AT181 (pTi,pAT); (B) PpS1239 (pMG1); (C) PpS1240 (pMG5); (D) JM125A$_2$; (E) C600 (RP4); (F) JC182.

Fig. 1-8. Agarose gel electrophoresis showing recovery of slowly-migrating DNA from three of four *E. coli* strains. The strains run in lanes (G) and (H) do not harbor plasmids. Electrophoresis was for 24 h at 60 V. (A) X1254; (B) PpS1239 (pMG1); (C) JM125A$_2$; (D) AT181 (pTi,pAT); (E) C600 (RP4); (F) PpS1240 (pMG5); (G) *E. coli* C; (H) JC182.

Fig. 1-9. Standard curve relating electrophoretic mobilities of size marker plasmids and JM125A_2 DNA molecules to their molecular weights. Horizontal lines represent one standard deviation to either side of the average normalized mobility.


E. coli chromosome (3)
pXX1 (7)
pAZ1 (12)
pAZ2 (18)
pAZ3 (20)
pXX2 (14)
pAZ5 (20)
pMG1 (17)
pAZ4 (20)
pMG5 (16)
F14 (5)
F14AF (5)
pAT (15)
pAZ6 (20)
pTi (15)
F (8)
pAZ7 (20)
RP4 (12)
MOLECULAR WEIGHT (MEGADALTONS)
1000
300
100
30
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
LOG NORMALIZED MOBILITY

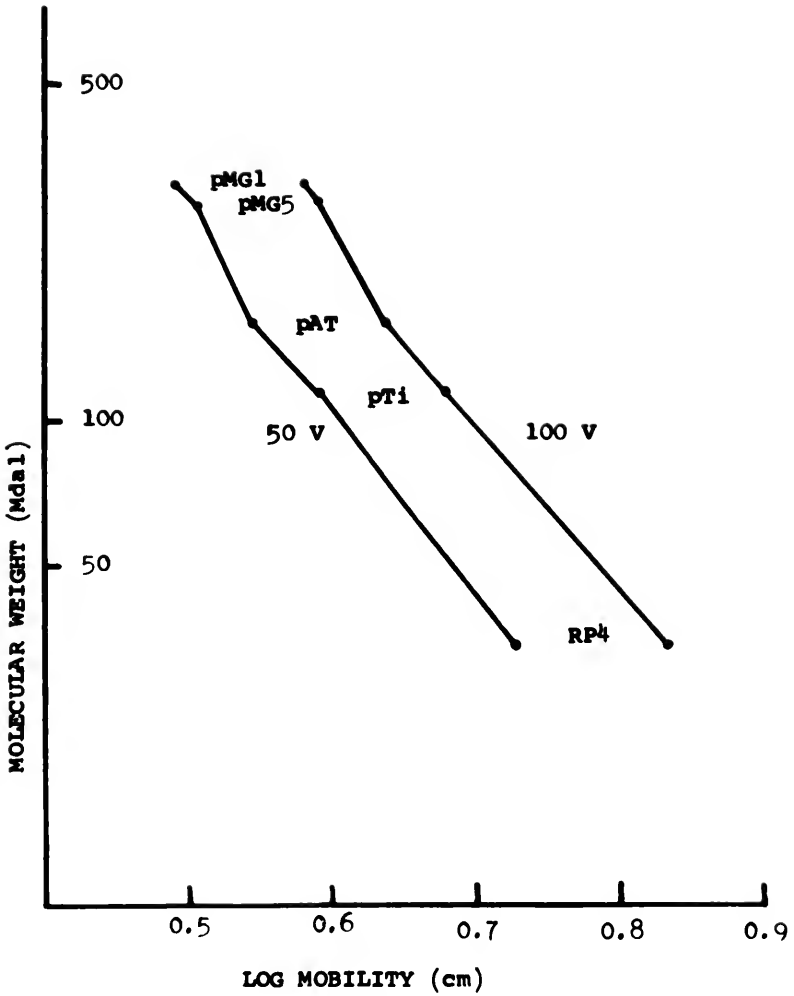


Fig. 1-10. Comparison of mobilities of standard plasmids in gels run at different voltages. The 50 V gel was run for 42 h and the 100 V gel for 24 h.

CHAPTER II
ACRIDINE ORANGE-INDUCED MUTATIONS IN *Azospirillum*

Introduction

Genetic material in the genus *Azospirillum* appears to be arranged in a manner unusual for bacteria. Rather than possessing a single large chromosome with or without accessory DNA in the form of plasmids, *Azospirillum* strains harbor unique arrays of up to eight different-sized covalently closed circular (CCC) DNA molecules, the largest estimated to be 1500 megadaltons (Mdal) (see Chapter I). The occurrence of so many molecules intermediate in size between what is normally considered to be very large for a plasmid (300 Mdal) and what would be considered a small chromosome (1500 Mdal) makes the usual plasmid/chromosome dichotomy less than obvious in these bacteria.

Plasmid DNA is generally defined as encoding functions not essential for cell growth under usual conditions. Growth of plasmid-harboring bacteria in the presence of acridine orange (AO) often permits the isolation, at an increased frequency, of strains which have been "cured" of the plasmid (30). We therefore grew a representative strain of *A. lipoferum* and one of *A. brasilense* in AO-containing medium and tested individual colonies for a variety of nonessential phenotypic traits. Since the capacity for

nitrogen fixation can be considered nonessential, the potentially mutant colonies were tested for growth on nitrogen-free medium. In addition, because of the blurring of the distinction between plasmid and chromosome in these bacteria, all colonies were screened for auxotrophy. Mutant colonies identified in this manner were analyzed for DNA content by agarose gel electrophoresis.

The present communication describes the successful correlation of three phenotypic properties with the presence of a specific plasmid in both Azospirillum strains examined. Furthermore, we report the isolation of a mutant having suffered multiple deletions in its DNA molecules and the isolation of both Nif^- and auxotrophic strains for which no obvious loss of DNA could be demonstrated.

Materials and Methods

Bacteria. A. brasilense JM125A_2 and A. lipoferum SpUSA5b were obtained from N. R. Krieg. Mutant strains derived from them are listed in Table 2-1.

Media. The succinate/mineral salts medium described by Okon et al. (50) was used for nitrogen-free growth. The same medium supplemented with 0.5% $(NH_4)_2SO_4$ was used as minimal agar (MA). Carbon-free agar (CFA) was MA lacking succinate. Carbon source utilization tests were done on MA with the appropriate carbon source replacing succinate at a concentration of 1%. Sensitivities of bacteria to inhibitors were determined on nutrient agar (Difco) supplemented with 0.01% yeast extract and filter-sterilized antibiotics or separately-autoclaved heavy metal salts.

Curing. Bacteria were treated with AO as described by Hirota (30), except that the pH was 8.0. The maximum concentration of AO which consistently permitted growth was 2.5 μg/ml.

Detection of DNA molecules. The electrophoretic method used to detect the various DNA molecules present in wild type and mutant strains has been extensively described elsewhere (see Chapter I).

Results

Table 2-1 lists the types of mutants isolated from cultures of A. brasilense JM125A$_2$ and A. lipoferum SpUSA5b grown in 2.5 μg/ml AO at pH 8.0 and the approximate frequencies with which they were found to occur in such cultures. No spontaneous mutants (from untreated cultures) were found.

Isolation of JM125A$_2$ mutants with the T phenotype. Originally, we screened JM125A$_2$ colonies derived from AO-treated cultures for a number of properties: 1) growth on minimal agar plates containing arabinose, galactose, ethanol, or butanol as sole carbon source; 2) sensitivity to trimethoprim (since wild type Azospirillum strains are resistant (3)); and 3) growth on succinate/mineral salts media with and without $(NH_4)_2SO_4$. No auxotrophic, Nif$^-$, Ara$^-$, Gal$^-$, or trimethoprim-sensitive mutants were found. However, mutants unable to grow on ethanol or butanol were isolated at a high frequency (Table 2-1). Further testing showed that acetate or acetaldehyde would support growth of the mutants.

Somewhat surprisingly, wild type colonies grew on the medium (intended as a control) to which no carbon source had been added. Growth on the carbon-free agar was slower and less luxurious than growth on any of the media containing added carbon. The mutant colonies failed to grow appreciably on the carbon-free medium. Although it appeared from these observations that Azospirillum was capable of autotrophic growth and that the mutants had lost this ability, both wild type and mutant strains produced a visible band of growth when a small inoculum (100 cells) was introduced into the carbon-free soft agar (0.02%) under a predominantly H_2/CO_2 atmosphere. Others have recently described autotrophic growth of Azospirillum (60).

Since heavy metal ion resistance is a characteristic often associated with plasmids in other bacteria (12, 42, 63, 71), we assessed the sensitivities of the mutant and wild type strains to the salts of seven heavy metals. No differential sensitivity to Ag^+, Cu^{2+}, Co^{2+}, Pb^{2+}, Hg^{2+}, or Ni^{2+} was observed; however, $3x10^{-5}$ M Cd^{2+} permitted growth of the wild type but not the mutant strains. Further analysis showed the mutants to be 30-fold more sensitive than the wild type to Cd^{2+} (Table 2-2). We have designated the "T" phenotype to represent the triad of cadmium sensitivity, inability to metabolize alcohols, and failure to grow on carbon-free agar under ambient atmosphere.

Loss of pAZ4 determines the T phenotype in JM125A$_2$. Figure 2-1 shows the DNA molecules of wild type and mutant JM125A$_2$ strains fractionated on an agarose gel as described (see Chapter I). All but one of the T mutants exhibited the DNA banding pattern represented by 125-T2 (lane D), which is identical to that of the wild type except for the absence of pAZ4 (cf. lanes A and D). In contrast, mutant strain 125-T1 appeared to have lost not only pAZ4 but pAZ7 and parts of pAZ2, pAZ3, pAZ5, and pAZ6 as well (lane B). Table 2-3 compares the estimated sizes of the 125-T1 DNA molecules to those of wild type JM125A$_2$.

We attempted to determine what functions, if any, could be ascribed to the DNA missing in 125-T1 but not in 125-T2. Wild type JM125A$_2$ and its T derivatives displayed similar sensitivities to UV light, and all reduced nitrate. As indicated in Table 2-2, however, 125-T2 was 10-fold and 125-T1 was 100-fold more sensitive to kanamycin than was the wild type. The sensitivities of the three strains to streptomycin were identical.

Isolation of a Nif$^-$ derivative of 125-T1. Despite repeated screening of colonies derived from nitrosoguanidine (NG) or AO-treated JM125A$_2$ cultures, we had never been able to isolate a mutant with the Nif$^-$ phenotype. The deletion of approximately 800 Mdal of DNA in 125-T1 (Table 2-3) suggested that at least 20% of the DNA in JM125A$_2$ is non-essential. This inference raised the possibility that our failure to isolate Azospirillum Nif$^-$ strains by conventional

mutagenesis might be due to occurrence of the nif genes in more than one copy. Therefore, we screened 2000 colonies derived from AO-treated 125-T1 cultures for growth on succinate/mineral salts medium with and without $(NH_4)_2SO_4$. One mutant was isolated which grew on the minimal medium only when nitrogen was supplied. This strain (125-T1N1) also failed to reduce acetylene even in the presence of low concentrations of $(NH_4)_2SO_4$ or yeast extract. The electrophoretic mobilities of its DNA molecules, however, could not be distinguished from those of its parent strain (Fig. 2-1, lanes B and C).

Mutants derived from AO-treated SpUSA5b cultures. We wondered whether types of mutants similar to the ones isolated from A. brasilense $JM125A_2$ could be isolated from A. lipoferum SpUSA5b. Only one of 2000 SpUSA5b colonies derived from AO-treated cultures displayed the T phenotype; thus, T mutants appear to occur at a somewhat lower frequency in SpUSA5b than in $JM125A_2$. An unexpected finding was the high frequency of Met^- auxotrophs (Table 2-1); no such mutants had been isolated from $JM125A_2$.

Figure 2-2 illustrates the DNA banding patterns of SpUSA5b and its mutant derivatives. As was the case with $JM125A_2$, the T phenotype correlated with loss of pAZ4 (lanes A and B). However, we did not observe obvious differences in electrophoretic mobility between the DNA molecules of wild type SpUSA5b and those of a representative Met^- derivative (lanes A and C).

125-T1N1 and USA-M1 are nonreverting. To investigate the possibility that 125-T1N1 and USA-M1 arose from AO-induced frameshift mutations, we attempted to isolate revertants of these strains from untreated, AO-treated, and NG-treated cultures. No true revertants were found. Slowly-growing colonies did appear at a frequency of approximately 10^{-8} when untreated 125-T1N1 was plated on nitrogen-free agar. However, when restreaked on fresh nitrogen-free plates they failed to flourish, and they were unable to reduce acetylene. Furthermore, their isolation frequency was enhanced to a much greater extent by NG than by AO.

A similar situation occurred with potential Met^+ revertants of USA-M1. Colonies arising on minimal agar grew when restreaked on fresh minimal plates, but growth on all types of media was slower than wild type growth. Like the pseudorevertants of 125-T1N1, their isolation frequency was increased significantly by NG but only marginally by AO. These Met^+ strains also displayed a deep reddish pigmentation, whereas colonies of our other SpUSA5b strains are peach colored.

Discussion

The results show that pAZ4-JM125A$_2$ and pAZ4-SpUSA5b determine, for their respective hosts, the Cad^R, Adh^+, and Cfa^+ phenotypes. The two plasmids are not entirely homologous, however, since the electrophoretic mobility of pAZ4-SpUSA5b is slightly lower than that of pAZ4-JM125A$_2$ (see Chapter I). Loss of pAZ4-JM125A$_2$ at a high frequency

in AO-treated JM125A$_2$ cultures is consistent with the known curing effects of acridine dyes (30). On the other hand, high-frequency AO-induced methionine auxotrophy and AO-induced multiple deletions in DNA have not been described.

The sensitivities of wild type and mutant Azospirillum strains to cadmium are, interestingly enough, comparable to the sensitivities exhibited by Cad^R and Cad^S Staphylococcus aureus strains (11). Recently, cadmium resistance in S. aureus has been shown to depend on an energy-dependent, plasmid-encoded efflux system (53, 69). A similar system may operate to confer cadmium resistance in Azospirillum. Alternatively, the configuration of membrane proteins in Azospirillum T mutants may result in a lesser degree of thiol group shielding than occurs in the wild type (58).

Growth of the T mutants on acetate and acetaldehyde but not on ethanol implies the existance of an alcohol dehydrogenase encoded by pAZ4-JM125A$_2$ and pAZ4-SpUSA5b. The basis for the inability of the T mutants to flourish on carbon-free agar under ambient atmosphere is less obvious. The defect does not appear to be in the capacity for autotrophy per se, as both mutant and wild type strains grow in carbon-free soft agar under an autotrophic atmosphere. Biosynthesis of ribulose diphosphate carboxylase in other bacteria has been shown to be repressed under conditions of high oxygen tension (37). Therefore, a single cell plated on carbon-free agar under ambient atmosphere cannot immediately begin to grow

autotrophically; it must instead utilize some intracellular carbon reserve to grow and divide several times, so that an aerobic layer of cells is formed under which a microaerophilic environment is created. Azospirillum strains do, in fact, accumulate large amounts of poly-ß-hydroxybutyrate (PHB), particularly (but not exclusively) when grown in nitrogen-free media (49). Although the T mutants as well as the wild type can be seen microscopically to contain PHB granules, it is possible that they have difficulty utilizing this storage polymer for growth.

The discovery that one of the T mutants (125-T1) had suffered multiple deletions in its DNA molecules is an observation which may be relevant to the proposed mode of action of AO in plasmid elimination. Some investigators have suggested that plasmids, owing to their relatively small size, are more accessible to AO than is the bacterial chromosome (62) or that AO selectively inhibits plasmid replication (31, 32). In contrast, others have maintained that AO causes nonspecific loss of DNA via inhibition of polymerase I (4, 5); since only cells having lost nonessential DNA survive, this nonspecific mode of action translates into an apparent specificity for plasmid (i.e. nonessential) DNA. Assuming that the generation of 125-T1 really was AO-mediated and not merely a spontaneous event which happened to occur in an AO-treated culture, our results tend to support the idea of a nonspecific interaction of AO with DNA. If AO specifically interacted with or inhibited replication of

plasmid DNA, one would not expect to observe partially deleted plasmids unless those plasmids were capable of dissociation into self-replicating component molecules. The possibility of four DNA molecules undergoing dissociation of this kind within the same cell seems remote.

Wild and mutant JM125A$_2$ strains displayed three levels of sensitivity to kanamycin, correlating with degree of DNA loss. This tends to implicate two or more proteins or one protein encoded by two or more loci (on at least two DNA molecules) in the determination of kanamycin sensitivity levels in Azospirillum. Furthermore, these proteins must act specifically on kanamycin and not on aminoglycosides in general, since the sensitivities of the various mutants to streptomycin were identical. Several types of aminoglycoside modifying enzymes have been described in other bacteria, but the mechanism by which the modified antibiotic confers resistance is not known (17). The mechanism of aminoglycoside uptake by sensitive cells is also obscure (17).

The Met$^-$ derivatives of SpUSA5b and the Nif$^-$ derivative of 125-T1 possess DNA molecules whose electrophoretic mobilities cannot readily be distinguished from those of their parent strains. These phenomena may be accounted for in one of three ways. First, the mutants might have arisen via the action of AO as a frameshift mutagen (15, 52). This possibility seems unlikely in view of the observed inability of AO to promote reversions in the mutant strains. Furthermore, the frameshift action of acridine dyes has been described

primarily in bacteriophage (15, 52); frameshift mutagenesis of bacteria at the frequencies reported here has not been described. A second possibility is that the mutants are deleted for all or part of a molecule not identified by the electrophoretic method used. This explanation also seems rather unlikely since we have used the method to identify molecules as large as the E. coli chromosome (see Chapter I). The third, and most likely, possibility is that the mutants carry deletions not large enough to lead to obvious differences in mobility for the affected molecules. Thus, 125-T1N1 might have arisen by deletion of the entire nif cluster from pAZ1-JM125A$_2$ (1500 Mdal); the wild type and mutant pAZ1-JM125A$_2$ molecules, differing in molecular weight by only 1%, would not be resolved by electrophoresis. We are currently attempting to determine whether this is the case by hybridizing a labeled nif probe to Southern blots (65) of fragmented mutant and wild type molecules.

Table 2-1. *Azospirillum* mutants isolated after growth in acridine orange

Parent Strain	Mutant	Phenotype[a]	Isolation frequency
$JM125A_2$	125-T1	$Adh^-Cfa^-Cad^SKan^S2$	$5x10^-4$
$JM125A_2$	125-T2	$Adh^-Cfa^-Cad^SKan^S1$	$5x10^-3$
125-T1	125-T1N1	$Adh^-Cfa^-Cad^SKan^S2Nif^-$	$5x10^-4$
SpUSA5b	USA-T100	$Adh^-Cfa^-Cad^SKan^S1$	$5x10^-4$
SpUSA5b	USA-M1	Met^-	$5x10^-3$

[a]Abbreviations:
Adh^- = No growth on ethanol or butanol
Cfa^- = No growth on minimal medium lacking added carbon
Cad^S = Sensitive to $3x10^-5$ M $CdCl_2$
Kan^S1 = Sensitive to 3 mg/ml kanamycin
Kan^S2 = Sensitive to 0.3 mg/ml kanamycin
Nif^- = No growth on nitrogen-free medium; no acetylene reduction
Met^- = Requires methionine for growth

Table 2-2. Sensitivities of wild type and mutant Azospirillum strains to cadmium, kanamycin, and streptomycin

Strain	Minimal inhibitory concentration		
	[Cd2+],M	[Km], μg/ml	[Sm],μg/ml
$JM125A_2$	10^{-4}	30	30
125-T1	$3x10^{-6}$	0.3	30
125-T1N1	$3x10^{-6}$	0.3	30
125-T2	$3x10^{-6}$	3	30

Table 2-3. Estimated sizes of DNA molecules found in $JM125A_2$ and 125-T1

Plasmid	Strain		deletion
	$JM125A_2$	125-T1	
pAZ1	1500	1500	-
pAZ2	1200	1100	100
pAZ3	640	430	210
pAZ4	400	-	400
pAZ5	360	320	40
pAZ6	130	95	35
pAZ7	46	-	46
total	4276	3445	831

Note: Sizes are in megadaltons. Sizes of $JM125A_2$ molecules were taken from Table 1-3. 125-T1 sizes were determined from mobilities in Fig. 2-1.

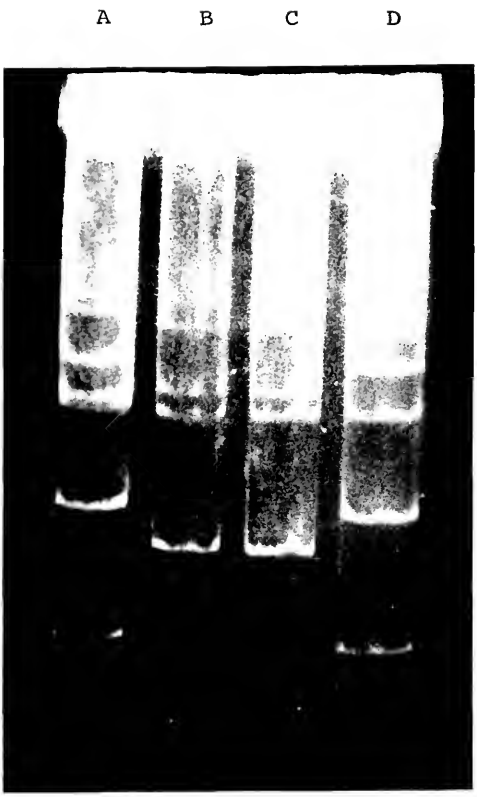


Fig. 2-1. Agarose gel electrophoresis showing DNA molecules recovered from wild type and mutant JM125A$_2$ strains: (A) JM125A$_2$; (B) 125-T1; (C) 125-T1N1; (D) 125-T2. Electrophoresis was for 23 h at 90 V.

Fig. 2-2. Agarose gel electrophoresis showing DNA molecules isolated from wild type and mutant SpUSA5b strains: (A) SpUSA5b; (B) USA-T100; (C) USA-M1. Electrophoresis was at 80 V for 31 h.

CHAPTER III
Nif GENE HYBRIDIZATION STUDIES OF *Azospirillum* DNA MOLECULES

Introduction

In previous chapters we showed that strains of *Azospirillum brasilense* and *A. lipoferum* harbor unique arrays of large circular DNA molecules and that phenotypically altered strains, some exhibiting a change in plasmid array, could be isolated at a high frequency from cultures treated with acridine orange. We now address the question of which of these molecules determines the phenotype of greatest general interest, i.e. nitrogen fixation. We had hoped that the Nif^- derivative of $JM125A_2$ would show a change in the mobility of one or more of its DNA molecules, but such was not the case. We therefore decided to try a different approach to the problem, based on molecular hybridization.

The structural genes for nitrogenase are thought to have been either introduced recently in evolutionary history into the various nitrogen-fixing bacteria or conserved to a greater extent than other translated prokaryotic genes (59). The basis for this view is the observation that *Klebsiella pneumoniae nif* structural genes can hybridize to DNA from all types of nitrogen-fixing prokaryotes, including both Gram-negative and Gram-positive bacteria, Actinomycetes, and Cyanobacteria (59). This interspecies homology has been exploited to study the organization of *nif* genes in

blue-green algae (39). Although Azospirillum DNA has not been examined for homology to Klebsiella nif DNA, there is no reason to think that Azospirillum would behave differently from other nitrogen-fixing prokaryotes in this regard, especially since the individual Azospirillum nitrogenase subunit proteins can form active complexes in vitro with complementary proteins from other nitrogen-fixing bacteria (23).

We obtained, from W. Klipp (36), an Escherichia coli strain harboring pWK27, a recombinant plasmid which includes an EcoRI fragment carrying the nifK, nifD, and nifH genes from K. pneumoniae. This molecule was isolated in large quantity, labeled to high specific activity by nick translation (57), and hybridized to Southern blots (65) of gels containing DNA molecules from wild type and mutant Azospirillum strains. The very limited success we have achieved with this technique indicates that pAZ1, the largest of the Azospirillum molecules, carries the nif structural genes in Azospirillum.

Materials and Methods

Bacterial strains. HB101(pWK27) was supplied by W. Klipp (36). Origins of Azospirillum strains are described in Table 1-1 (Chapter I) and Table 2-1 (Chapter II).

Isolation of pWK27. To one liter of cell culture grown at 37°C to Klett 90 was added 170 mg chloramphenicol. The cells were incubated at 37°C for 20 h, harvested, washed with 10 mM NaCl, and resuspended in 6 ml 0.02 M EDTA, 0.025

M Tris, 0.9% glucose (pH 8.0). Lysozyme (12 mg) was added and the cells were chilled on ice for 30 min. Next, 12 ml 0.08% NaOH, 0.8% SDS was added and the lysate was gently swirled for 5 min. Following addition, with gentle mixing, of 9 ml KAc (pH 4.8), the lysate was incubated on ice for 2 h. The lysate was then centrifuged at 15,000 rpm for 30 min and the supernatant solution was transferred to a fresh tube. The addition of PEG 6000 to a final concentration of 10% followed by incubation on ice for 2 h caused the plasmid to precipitate and it was pelleted at 2,500 rpm for 5 min.

The pellet was resuspended in 2 ml 50mM Tris, 1mM EDTA (pH 8.0). Ribonuclease was added to a concentration of 5 μg/ml and the solution was incubated at 37°C for 30 min. Following adjustment of the volume to 10 ml with 50 mM Tris, 1mM EDTA (pH 8.0), the solution was extracted twice each with phenol/chloroform, chloroform, and ether. Residual ether was blown off with air. The plasmid was then ethanol precipitated, washed in 70% ethanol, and further purified by dye bouyant density ultracentrifugation. Plasmid bands, visualized with UV light, were removed from the centrifuge tubes with a plastic syringe. Ethidium bromide was removed by extraction with isopropanol and CsCl by dialysis against three changes (2 l each) of 25 mM Tris, 1 mM EDTA.

Blotting. Agarose gels containing separated Azospirillum DNA molecules were prepared as described in Chapter I. DNA from the gels was blotted onto strips of nitrocellulose according to the method of Wahl et al. (70). This is a

slight modification of Southern's original technique (65). The gels are treated with 0.25 M HCl as an initial step in order to partially depurinate the DNA and fragment it for more efficient transfer.

Nick translation of pWK27. The probe was labeled with ^{32}P according to the method described by Rigby et al. (57). One hundred microCuries $^{32}P\alpha$-dCTP was dried under vacuum in a 1.5 ml microfuge tube. To this was added 5 µl 0.5 M Tris, 0.1 M β-mercaptoethanol, 0.05 M $MgCl_2$; 24 µl of a 1:1:1 dNTP mix (200 mM each); 1 µg pWK27 DNA; and water to make 48 µl total volume. After 5 min at 30°C, 1 µl diluted, activated DNase I was added. (DNase was activated by diluting a 1 mg/ml stock in 10 mM HCl 1:2000 into 10 mM Tris, 5 mM $MgCl_2$, 1 mg/ml BSA (pH 7.6)). After 2 min at 30°C, 0.5 µl DNA polymerase I (5 U/ml) was added and the reaction was held at 15°C for 1 h, whereupon it was terminated by the addition of 5 µl 0.25 M EDTA. The reaction mixture was extracted with phenol and the labeled plasmid was separated from the unincorporated nucleotides by passage through a Sepharose 4B or Sephadex G-100 column or by electrophoresis through a 0.7% low melting point agarose gel.

Hybridization. All hybridizations and prehybridizations were carried out in Sears Seal-n-Save plastic bags. Filters were prehybridized for a minimum of 12 h at 65°C in a solution consisting of 0.5% SDS, 20 µg/ml denatured salmon sperm DNA, 10x Denhardt's solution (20), 2.5x SSC, and 0.05 M Na_2HPO_4 (pH 8.0). Hybridizations were carried out under the

same conditions except that 1x Denhardt's solution was used and denatured, labeled pWK27 was included. The washing protocol of Thomashow et al. was followed (68). This consists of one 30 min wash with 2.5x SSC at room temperature followed by four washes (30 min each) with 2.5x SSC at 65°C and one wash at 65°C with 0.1x SSC.

Autoradiography. The dried filters were exposed to X-ray film at -70°C in the presence of an intensifying screen. Exposure times were 2 h for the control hybridization of pWK27 to its restriction fragments and a minimum of 1 week for the Azospirillum hybridizations.

Results and Discussion

Table 3-1 summarizes the variations in protocol and outcomes of five hybridization experiments. Both Fig. 3-2 and Fig 3-4 were taken from Experiment 2. Despite the somewhat disappointing results, we can conclude from Fig. 3-4 that the Azospirillum nif structural genes are carried on pAZ1, the molecule which probably represents the chromosome of these bacteria.

Since the control hybridization (Fig. 3-2) gave such a strong signal it is doubtful that our difficulty in detecting Azospirillum nif sequences reflects a serious flaw in experimental procedure. Rather, the problem seems to be one of sensitivity; the autoradiogram illustrated in Fig. 3-4 required 8 days exposure and shows only faint bands of hybridization. There would appear to be two explanations for this low level of hybridization. First, assuming that

the Azospirillum nif genes are carried on pAZ1, less than 0.5% of the DNA in the band representing pAZ1 is capable of hybridizing to the probe. Second, since there is some degree of divergence in the DNA sequences of nif structural genes in various bacteria (59), the probe DNA is not entirely homologous to that portion of pAZ1 which is capable of hybridization. DNA preparations from different strains of Rhizobium hybridize with differing intensities to Klebsiella nif DNA (59). We do not think that the low level of hybridization exhibited by Azospirillum DNA reflects poor transfer from gel to nitrocellulose. Gels were always restained and examined for DNA after transfer; none was ever found.

Table 3-1. Summary of results of hybridization experiments

Expt.	Specific activity of probe[a]	Labeled nucleotide	Purification of probe	Method of DNA transfer	Hybridization?
1	1.6×10^8	dCTP	Sepharose 4B	Southern	no
2	9.1×10^7	dCTP	Sepharose 4B	Southern	yes
3	2.2×10^7	dCTP	Sepharose 4B	electroblot	no
4	1.8×10^7	dCTP	Sephadex G-100	electroblot	no
5	2.2×10^7	dCTP	electrophoresis	Southern	no

[a]cpm per μg DNA

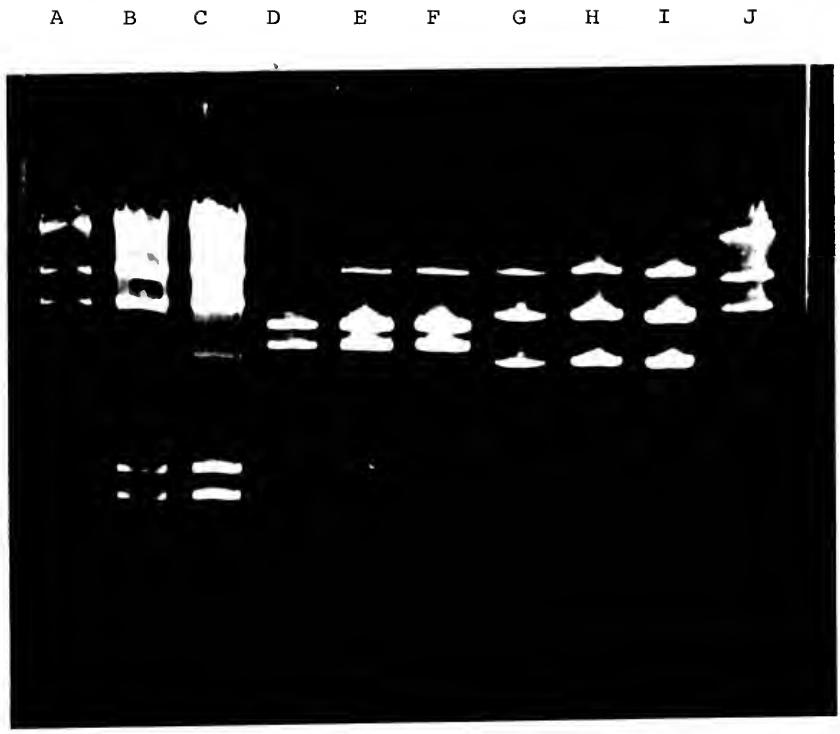


Fig. 3-1. Agarose gel electrophoresis of pWK27 digested with HindIII (D-F) or EcoRI (G-I). Some undigested OC and CCC plasmid DNA is visible. Lanes (A-C) and (J) show lambda HindIII fragments used as size standards. Volumes of DNA solutions added to wells were as follows: (A) 1 ul; (B,J) 2 ul; (C) 4 ul; (D,G) 3 ul; (E,H) 6 ul; (F,I) 9 ul. Electrophoresis was at 60 V for 4 h.

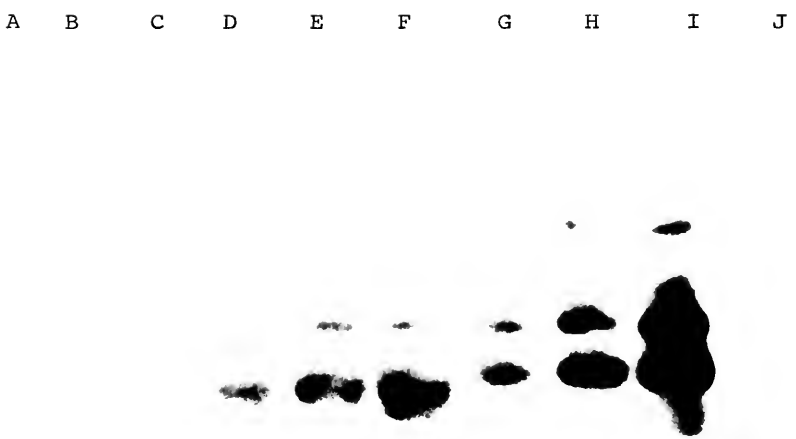


Fig. 3-2. Autoradiogram of gel shown in Fig. 3-1. Gel was blotted and hybridized to labeled pWK27 as described in the text. Exposure was for 2 h. (A-C,J) HindIII-digested lambda; (D-F) HindIII-digested pWK27; (G-I) EcoRI-digested pWK27.

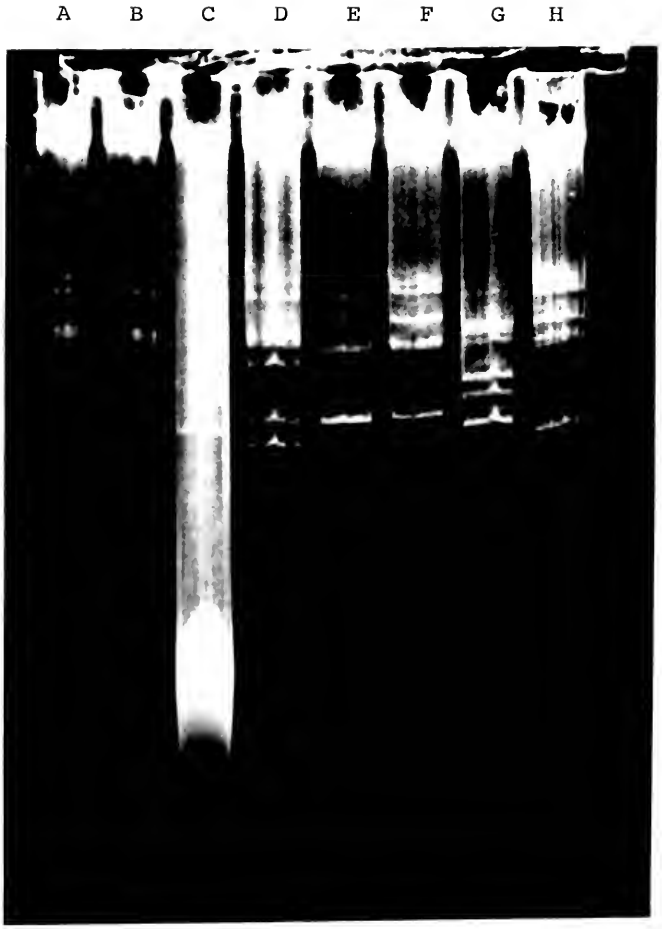


Fig. 3-3. Agarose gel electrophoresis showing DNA molecules recovered from Azospirillum strains of diverse geographic origin. Electrophoresis was for 30 h at 80 V. A. brasilense strains: (A) Sp7; (B) Sp13t; (C) Sp84; (D) Cd; (E) JM82A$_1$; (F) JM125A$_2$. A. lipoferum strains: (G) SpUSA5b; (H) SpRG6xx.

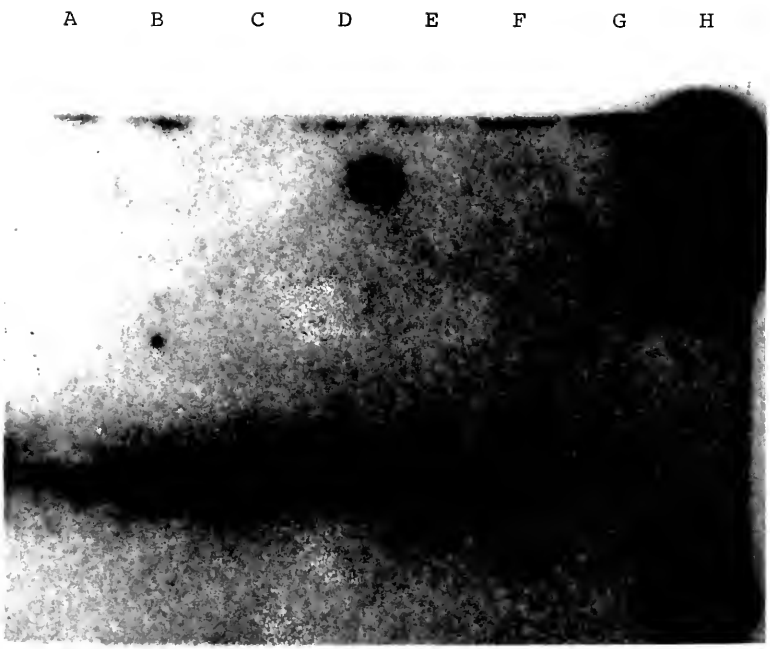


Fig. 3-4. Autoradiogram of gel shown in Fig. 3-3. The gel was trimmed of material above pAZ1, blotted onto nitrocellulose, and hybridized to labeled pWK27 as described in the text. Exposure was for 8 days. (A) Sp7; (B) Sp13t; (C) Sp84; (D) Cd; (E) $JM82A_1$; (F) $JM125A_2$; (G) SpUSA5b; (H) SpRG6xx.

APPENDIX A
NORMALIZATION OF GEL MOBILITY DATA

In order to calculate a regression line relating electrophoretic mobility to molecular weight, it was necessary to pool mobility data from 20 gels run for various lengths of time under slightly differing conditions. Therefore, the mobility data from 19 of the gels had to be normalized to the data from one gel chosen as a standard. The gel illustrated in Fig. 1-6 was chosen as the standard because it includes the greatest number (15) of DNA bands.

The standard gel was designated B and the mobilities of its DNA bands were B_1, B_2,...., B_n. The mobilities of the bands in gel A (which had in common with gel B n different plasmid bands numbered 1,2,...,n) were designated A_1, A_2,..., A_n. The constant K_a was calculated for which the expression

$$\Sigma \left| K_a A_i - B_i \right| / (K_a A_i + B_i)$$

where $i=1,2,\ldots,n$

was at a minimum. Then the mobilities of the plasmids in gel A were multiplied by K_a. A different K_a was calculated for each gel to be normalized.

APPENDIX B
REGRESSION LINE CALCULATIONS

If the logarithms of the average normalized mobilities of the standard plasmids were designated as X_1, X_2,...X_n and the logarithms of their molecular weights (in megadaltons) were designated Y_1, Y_2,...Y_n then the logarithm of the molecular weight (Y_t) of the unknown molecule could be calculated from the logarithm of its mobility (X_t) according to the formula

$$Y_t = A+BX_t$$

where $A=\overline{Y}-B\overline{X}$

$$B = \frac{\Sigma X_i Y_i - \frac{1}{n}(\Sigma X_i)(\Sigma Y_i)}{\Sigma X_i^2 - \frac{1}{n}(\Sigma X_i)^2}$$

$$\overline{X} = \Sigma X_i/n$$

$$\overline{Y} = \Sigma Y_i/n$$

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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